Short Communication

Calcium, Vitamin D, and Apoptosis in the Rectal Epithelium

Eric A. Miller,[1] Temitope O. Keku,[1,3] Jessie A. Satia,[2,4] Christopher F. Martin,[1,3] Joeseph A. Galanko,[3] and Robert S. Sandler[3]

Departments of [1]Epidemiology and [2]Nutrition, School of Public Health, [3]Department of Medicine and Center for Gastrointestinal Biology and Disease, School of Medicine, University of North Carolina, Chapel Hill, North Carolina; and [4]Amgen, Inc., Thousand Oaks, California

Abstract

Objective: **Decreased apoptosis in the colon is potentially an early indicator of colon cancer risk and may be influenced by calcium and vitamin D. This report describes the associations of calcium intake and 25-hydroxyvitamin D levels with apoptosis in colorectal epithelium.**

Methods: **Consecutive patients undergoing colonoscopies were recruited for a study designed to examine risk and etiologic factors for colorectal adenomas. Diet was assessed by food frequency questionnaire, and in one subpopulation, serum 25-hydroxyvitamin D levels were measured using an enzyme immunoassay. Apoptosis was scored from normal rectal mucosal pinch biopsies. Linear and logistic regression analyses were used to examine associations between calcium, serum vitamin D, and apoptotic scores. Data were available for 498 and 280 patients for the calcium and vitamin D analyses, respectively.**

Results: **Associations of calcium intake and vitamin D with apoptosis were modified by adenoma case-status. In an adjusted logistic regression model, patients with adenomas in the highest versus lowest tertile of dietary calcium intake had 3.4 times higher odds [95% confidence interval (CI), 0.9-12.9] of elevated apoptotic scores. In adenoma-free patients, high calcium intake was not related to apoptosis (OR, 1.2; 95% CI, 0.6-2.7). In contrast, the highest level of 25-hydroxyvitamin D was associated with higher apoptosis in adenoma-free patients (OR, 2.6; 95% CI, 1.1-6.2) and slightly lower levels in patients with adenomas (OR, 0.6; 95% CI, 0.2-2.2).**

Conclusion: **These results are consistent with a calcium and vitamin D-mediated apoptotic mechanism in colon carcinogenesis.** (Cancer Epidemiol Biomarkers Prev 2005; 14(2):525–28)

Introduction

Apoptosis is essential to maintaining the structure of the colorectal epithelial crypts by counterbalancing cellular proliferation. Inhibition of apoptosis is an important step in colorectal carcinogenesis, where the shift in balance facilitates the clonal expansion of cancerous cells. There is evidence that a decreased level of apoptosis in the colorectal epithelium may be an early indicator of increased risk of adenoma development or colorectal cancer (1-3). In addition, several studies have shown that apoptotic levels may be influenced by dietary and chemopreventive agents (4-6).

Calcium and vitamin D are biologically linked and both have shown promise as preventive agents of colorectal cancer (7-11). Animal models and cell culture have provided evidence that these micronutrients may act by influencing apoptosis. For example, compared with mice on a standard diet of 0.5% calcium, increasing the concentration to 1.0% resulted in higher levels of apoptosis in the distal colorectal epithelium (12). In human colorectal adenoma and cancer cell lines, the active metabolite of vitamin D [1,25 $(OH)_2D_3$] was found to induce apoptosis in a dose-dependent manner (13).

Although calcium and vitamin D have been shown to increase apoptosis in animal models and cell culture, to date, no study has examined their association with apoptosis in human colorectal epithelium. Data from a cross-sectional study of patients undergoing colonoscopic exams were used to examine whether calcium and vitamin D are associated with increased apoptosis in normal rectal biopsy tissue.

Materials and Methods

Study Design. A more detailed description of the Diet and Health Study III population and measurement of apoptosis has been published previously (14). In brief, consecutive patients undergoing diagnostic or screening colonoscopies at the University of North Carolina Hospitals in Chapel Hill between August 1, 1998 and March 4, 2000 were recruited to participate in the study. In order to be eligible, participants had to be at least 30 years old, proficient in the English language, and without colitis, a history of familial polyposis, previous colonic resection, previous colon cancer, or adenoma.

Of the 2,452 colonoscopies done during the study period, 926 (38%) patients were eligible for the study and 803 (93.4%) consented to participate. Eligible patients were also asked to consent to six rectal mucosal pinch biopsies taken by the colonoscopist during the exam. Adequate specimens were obtained from 504 (63%) patients. After exclusion for incomplete data (n = 6), there were 498 (62%) patients (174 patients with adenomas and 324 non-adenoma patients) available for this analysis.

Lifestyle and dietary questionnaires were completed over the telephone by an interviewer blinded to participant case-status. A Block food frequency questionnaire (15), modified to include an additional 29 foods common to diets in North

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Requests for reprints: Robert S. Sandler, Department of Medicine and Center for Gastrointestinal Biology and Disease, School of Medicine, CB#7555, 4111 Bioinformatics Building, University of North Carolina, Chapel Hill, NC 27599-7555. Phone: 919-966-0090; Fax: 919-966-2478. E-mail: robert_sandler@med.unc.edu

Carolina (16), was used to assess patients' usual diet during the year preceding the colonoscopy. Dosage (e.g., 100, 250, 500, and 1,000 mg), frequency (days/week), and duration (years) of use of several vitamin and mineral supplements, including calcium, were collected as well. Estimated daily dietary and supplemental intakes were calculated by the Block food frequency analysis program.

Serum 25-Hydroxyvitamin D Assay. Due to limited quantity, serum from fasting blood samples was available for only 92 (53%) patients with adenomas and 188 (58%) non-adenoma patients of this subpopulation. Blood samples were taken at the time of colonoscopy and kept at 4°C. Serum was separated within 2 hours and frozen at −80°C. Serum 25-hydroxyvitamin D levels were measured using an enzyme immunoassay (ALPCO Diagnostics, Windham, NH) with a reported range of 2.5 to 100 ng/mL. Samples were assayed in duplicate, assigning the average concentration to each patient.

Measurement of Apoptosis. Apoptosis was scored by an experienced laboratory technician using standard morphologic criteria on H&E stained slides and by the terminal nucleotidyl transferase–mediated nick end labeling method. Two biopsies from each patient were examined, using five sections per biopsy specimen, 50 μm apart. This is equivalent to ~8 to 12 longitudinal crypts scored per biopsy. Crypt selection criteria were described previously (17). We observed a positive correlation (r = 0.55, P < 0.0001) between the two methods of measuring apoptosis. Because problems with the terminal nucleotidyl transferase–mediated nick end labeling assay have been previously noted (18) and because the relationship between calcium, vitamin D, and apoptosis were similar for each method, only the results using morphologic criteria are presented here. Apoptotic cells were determined by the presence of observed cell shrinkage, chromatin contraction, and nuclear fragmentation. The mean apoptosis score was calculated as the mean number of apoptotic cells per crypt. Selected slides were scored independently a second time with a calculated reproducibility of 99%.

Table 1. Patient characteristics by adenoma case-status

	Adenoma case (n = 174)	Non-adenoma patients (n = 324)
Mean apoptosis score (SD)	2.5 (0.6)	2.9 (0.7)
Male (%)	91 (52.6)	107 (33.0)
White non-Hispanic (%)	115 (72.3)	239 (77.9)
Mean age (SD)	59.8 (10.2)	54.0 (11.2)
Family history of colon cancer (%)	41 (23.6)	101 (31.2)
Ever smoker, >100 cigarettes (%)	97 (56.1)	157 (48.5)
Current smoker (%)	28 (16.1)	63 (19.5)
Regular nonsteroidal anti-inflammatory drugs use (%)*	74 (42.5)	168 (51.9)
Regular calcium supplement use (%)*	54 (31.0)	110 (34.0)
Mean dietary calcium intake (SD)	620.6 (363.3)	671.2 (420.9)
Mean 25-(OH) vitamin D (ng/mL; SD)†	28.5 (16.8)	32.0 (17.5)
Mean body mass index (kg/m^2; SD)	28.3 (6.0)	27.6 (6.4)
Mean total energy intake (kcal/d; SD)	1,546.4 (643.8)	1,555.9 (655.2)
Mean energy from fat (%)	35.2	34.4

*Defined as ≥15 times per month.
†In 92 patients with adenomas and 188 without adenomas.

Table 2. Associations of calcium intake and serum 25-hydroxyvitamin D with apoptosis, categorized above and below the median, overall, and by adenoma case status

	Overall, OR (95% CI)	Adenoma case, OR (95% CI)	Non-adenoma patient, OR (95% CI)
Total calcium*			
≤533.8 (mg/d)	1.0	1.0	1.0
533.9-1,102.1	1.5 (0.8-2.7)	2.3 (0.8-6.9)	1.2 (0.6-2.4)
>1,102.1	1.2 (0.7-2.2)	1.7 (0.6-5.3)	1.0 (0.5-2.1)
Dietary calcium*			
≤419.6 (mg/d)	1.0	1.0	1.0
419.6-739.1	1.4 (0.8-2.4)	3.5 (1.1-11.2)	1.0 (0.5-2.0)
>739.1	1.7 (0.9-3.2)	3.4 (0.9-12.9)	1.2 (0.6-2.7)
Serum 25-(OH) vitamin D†			
≤20.9 (ng/mL)	1.0	1.0	1.0
20.9-34.9	1.8 (0.9-3.6)	0.6 (0.2-1.9)	3.6 (1.5-9.1)
>34.9	1.5 (0.7-2.9)	0.6 (0.2-2.2)	2.6 (1.1-6.2)

NOTE: Median = 2.74 cells per crypt.
*Adjusted for race, sex, adenoma case status, total caloric intake, total fiber intake, and total vitamin C intake.
†Conducted in 92 patients with adenomas and 188 without adenomas and adjusted for adenoma case-status, race, and date of blood draw.

Data Analysis. Unadjusted and multivariable logistic regression were used to calculate the odds ratio (OR) and corresponding 95% confidence interval (CI) of increased calcium intake and serum 25-hydroxyvitamin D levels with apoptosis, categorized above versus below the median, using the median from the total study population (median = 2.74 cells per crypt; refs. 14, 19). Using continuous apoptosis scores, least-squared means and corresponding 95% CIs were calculated from linear regression models to estimate mean apoptosis scores by tertiles of calcium and vitamin D.

We examined effect measure modification by age (30-50, 51-60, 61-70, >70 years), race (non-Hispanic White or non-Hispanic Black), sex, fat intake (above or below the median), and adenoma case status. Modification of the association between calcium and apoptosis by vitamin D was explored, however, the study population was undersized to adequately assess the effect measure modification. The likelihood ratio test or 100% difference in stratified estimates were used as criteria of effect measure modification with logistic regression methods.

Using backward elimination, the variables assessed for confounding in these analyses were fat intake (continuous), total energy intake (continuous), fiber intake (continuous), use of nonsteroidal anti-inflammatory drugs (regular use defined as ≥15 times per month; infrequent or non-use 0-15 times per month), smoking status (current, non-current; ever, never), first-degree family history of colon cancer (yes or no), adenoma case status, sex, age (continuous), race, and body mass index (≥30 or <30 kg/m^2). A 10% change in OR was used as criteria for confounding in logistic regression models.

Results

Approximately 60% of the patients were female and 24% were self-reported non-Hispanic Black, with a mean age of 60.0 years (SD, 11.2). Patients with adenomas accounted for 35.2% (n = 174) of the study population and were more likely to be older, male, and ever-smokers (Table 1). The distributions were not statistically different in the subpopulation for which vitamin D was measured (data not shown) except by race, where fewer Black, non-Hispanics had available serum compared with White, non-Hispanics (18.1% versus 24.8%; P = 0.03).

Table 2 presents the adjusted associations between calcium and 25-hydroxyvitamin D with levels of apoptosis

Table 3. Estimated least-squared means of apoptotic scores by tertiles of total calcium intake, dietary calcium intake, and serum 25-hydroxyvitamin D

	Mean apoptotic score		
	Overall, (95% CI)	Adenoma Case, (95% CI)	Non-adenoma patient, (95% CI)
Total calcium*			
≤533.8 (mg/d)	2.71 (2.59-2.83)	2.34 (2.17-2.52)	2.91 (2.75-3.08)
533.9-1,102.1	2.83 (2.73-2.94)	2.61 (2.45-2.77)	2.95 (2.81-3.10)
>1,102.1	2.78 (2.66-2.90)	2.44 (2.27-2.61)	2.95 (2.80-3.11)
Dietary calcium*			
≤419.6 (mg/d)	2.70 (2.57-2.82)	2.34 (2.15-2.53)	2.88 (2.71-3.05)
419.6-739.1	2.79 (2.68-2.89)	2.49 (2.33-2.65)	2.96 (2.82-3.10)
>739.1	2.84 (2.72-2.96)	2.59 (2.40-2.77)	2.98 (2.82-3.13)
Serum 25-(OH) vitamin D†			
≤20.9 (ng/mL)	2.69 (2.56-2.82)	2.58 (2.37-2.79)	2.73 (2.57-2.90)
20.9-34.9	2.88 (2.74-3.02)	2.56 (2.33-2.78)	3.05 (2.87-3.23)
>34.9	2.82 (2.67-2.97)	2.50 (2.25-2.75)	2.97 (2.78-3.16)

NOTE: Regression models adjusted for race, sex, total caloric intake, vitamin C intake, and fiber intake and adenoma case-status.

*Regression models adjusted for race, sex, total caloric intake, vitamin C intake, fiber intake, and adenoma case-status.

†Conducted in 92 patients with adenomas and 188 patients without adenomas; regression models adjusted for time of blood draw, race, and adenoma case-status.

above the median, overall, and stratified by adenoma case-status. Overall, there was a small, nonstatistically significant association between dietary calcium and apoptosis above the median. However, the association was substantially stronger in patients with adenomas compared with those without. Although imprecise, patients with adenomas and dietary calcium intake in the second and third tertiles were associated with a more than three times higher odds of apoptotic scores above the median compared with those in the lowest tertile. There was no association in patients without adenomas (likelihood ratio test; $P = 0.17$).

Higher levels of serum vitamin D were also associated with a small, nonstatistically significant, increased odds of apoptosis above the median (Table 2). When stratified by adenoma case-status, high serum vitamin D was strongly associated with apoptosis above the median in patients without adenomas and inversely associated in patients with adenomas (likelihood ratio test; $P = 0.13$).

In linear regression models adjusted for adenoma case-status, race, sex, vitamin C, fiber, and total energy, estimated mean apoptotic scores increased with higher dietary calcium intake in patients with adenomas but not adenoma-free patients (Table 3). However, the association between calcium and apoptosis was not statistically significant in the adjusted model ($P = 0.30$). When vitamin D was categorized into tertiles, in agreement with the categorical analysis (Table 2), the biggest increase in estimated mean apoptotic scores was between the first and second tertiles and the association was stronger in patients without adenomas (Table 3).

Discussion

There is increasing evidence suggesting that calcium and vitamin D play important roles in reducing the risk of colorectal cancer. In agreement with studies using animal models and cell culture (12, 13), our results support a mechanism in which calcium and vitamin D reduce the risk of colorectal cancer by increasing the level of apoptosis in the colorectal epithelium. However, for calcium, the positive association was stronger in patients with adenomas, whereas for vitamin D, the association was stronger in adenoma-free patients.

Although estimates were imprecise, calcium was strongly associated with high levels of apoptosis in patients with adenomas using logistic regression. However, the relative lack of association between calcium and apoptosis in linear regression should prompt caution in assessing the strength of the association. Nonetheless, the linear increase in predicted mean apoptotic scores by increasing tertiles and quartiles of dietary calcium for patients with adenomas, but not for adenoma-free patients, was somewhat reassuring.

Interestingly, supplemental plus dietary calcium intake was less strongly associated with apoptosis compared with dietary intake alone. It is unlikely that the stronger results for dietary versus total calcium intake could be due to doses exceeding the most effective range because the levels of calcium intake in this population were low compared with previous studies and because trials to prevent recurrent adenomas supplemented 1,200 and 2,000 mg of calcium per day (7, 8). Different associations for total, dietary, and supplemental calcium intake have been reported in other studies (20, 21), but reasons for the disparity remain unclear.

We also found evidence that higher serum levels of 25-hydroxyvitamin D were independently associated with higher apoptotic scores. In contrast to calcium intake, the positive association was limited to adenoma-free patients. There is very little published data examining the association between vitamin D and apoptosis in the colorectal epithelium. Our results are consistent with the two other studies that examined the association between the functional form of vitamin D, 1,25-dihydroxyvitamin D, and apoptosis in colorectal adenoma and cancer cell lines (13, 22). However, vitamin D metabolites have also been found to induce apoptosis in other tissues and cancerous cell types, such as breast and prostate cancer cells (23, 24).

Previous studies have found that calcium's association with proliferation and normalization within the colorectal epithelial crypt was dependent on serum 25-hydroxyvitamin D level. It has also been shown that the intercellular calcium gradient is dependent on dietary intake of vitamin D in colonic crypts isolated from mice (25). Vitamin D, along with other functions, regulates calcium homeostasis, and if calcium reduces the risk of colorectal cancer by increasing apoptosis, it is likely influenced by vitamin D as well. Unfortunately, we did not have a sufficient number of patients with serum vitamin D measurements to adequately assess the interactive effects of calcium and vitamin D on apoptosis.

Although apoptosis was measured in normal rectal tissue, a statistically significant lower level of apoptosis in the normal rectal tissue of patients with adenomas compared with those without was previously shown in this study population (19). This has been described as a ''field effect'' and other studies have reported this phenomenon as well (2, 3). Despite demonstrating intraindividual variation in levels of apoptosis within different locations of the colon, patients with colorectal adenomas or cancer have been shown to have substantially lower overall apoptosis levels in normal tissue than adenoma-free patients (3).

Importantly, the study population was diverse with good heterogeneity by age, race, and sex. In addition to the use of serum vitamin D measurement, extensive data on demographic characteristics, diet, and lifestyle, allowed us to assess and adjust for several variables that could confound the associations.

Calcium and vitamin D have exhibited considerable potential to decrease the risk of colorectal cancer. Although both have been implicated in several biological processes that could affect cancer risk, the mechanism by which calcium and vitamin D reduce the risk remains uncertain.

Although dependent on adenoma case-status, our study is consistent with animal and cell culture data which suggests that the reduction in risk may be, at least in part, attributable to increasing the level of apoptosis in the colon.

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